

strands. Several of the known DNAPs contain associated nuclease activities in addition to the synthetic activity of the enzyme.

Some DNAPs are known to remove nucleotides from the 5' and 3' ends of DNA chains [Kornberg, *DNA Replication*, W.H. Freeman and Co., San Francisco, pp. 127-139 (1980)]. These nuclease activities are usually referred to as 5' exonuclease and 3' exonuclease activities, respectively. For example, the 5' exonuclease activity located in the N-terminal domain of several DNAPs participates in the removal of RNA primers during lagging strand synthesis during DNA replication and the removal of damaged nucleotides during repair. Some DNAPs, such as the *E. coli* DNA polymerase (DNAPEc1), also have a 3' exonuclease activity responsible for proof-reading during DNA synthesis (Kornberg, *supra*).

A DNAP isolated from *Thermus aquaticus*, termed *Taq* DNA polymerase (DNAPTaq), has a 5' exonuclease activity, but lacks a functional 3' exonucleolytic domain [Tindall and Kunkell, *Biochem.* 27:6008 (1988)]. Derivatives of DNAPEc1 and DNAPTaq, respectively called the Klenow and Stoffel fragments, lack 5' exonuclease domains as a result of enzymatic or genetic manipulations [Brutlag *et al.*, *Biochem. Biophys. Res. Commun.* 37:982 (1969); Erlich *et al.*, *Science* 252:1643 (1991); Setlow and Kornberg, *J. Biol. Chem.* 247:232 (1972)].

The 5' exonuclease activity of DNAPTaq was reported to require concurrent synthesis [Gelfand, *PCR Technology - Principles and Applications for DNA Amplification* (H.A. Erlich, Ed.), Stockton Press, New York, p. 19 (1989)]. Although mononucleotides predominate among the digestion products of the 5' exonucleases of DNAPTaq and DNAPEc1, short oligonucleotides (≤ 12 nucleotides) can also be observed implying that these so-called 5' exonucleases can function endonucleolytically [Setlow, *supra*; Holland *et al.*, *Proc. Natl. Acad. Sci. USA* 88:7276 (1991)].

In WO 92/06200, Gelfand *et al.* show that the preferred substrate of the 5' exonuclease activity of the thermostable DNA polymerases is displaced single-stranded DNA. Hydrolysis of the phosphodiester bond occurs between the displaced single-

stranded DNA and the double-helical DNA with the preferred exonuclease cleavage site being a phosphodiester bond in the double helical region. Thus, the 5' exonuclease activity usually associated with DNAPs is a structure-dependent single-stranded endonuclease and is more properly referred to as a 5' nuclease. Exonucleases are enzymes which cleave nucleotide molecules from the ends of the nucleic acid molecule. Endonucleases, on the other hand, are enzymes which cleave the nucleic acid molecule at internal rather than terminal sites. The nuclease activity associated with some thermostable DNA polymerases cleaves endonucleolytically but this cleavage requires contact with the 5' end of the molecule being cleaved. Therefore, these nucleases are referred to as 5' nucleases.

When a 5' nuclease activity is associated with a eubacterial Type A DNA polymerase, it is found in the one-third N-terminal region of the protein as an independent functional domain. The C-terminal two-thirds of the molecule constitute the polymerization domain which is responsible for the synthesis of DNA. Some Type A DNA polymerases also have a 3' exonuclease activity associated with the two-third C-terminal region of the molecule.

The 5' exonuclease activity and the polymerization activity of DNAPs have been separated by proteolytic cleavage or genetic manipulation of the polymerase molecule. To date thermostable DNAPs have been modified to remove or reduce the amount of 5' nuclease activity while leaving the polymerase activity intact.

The Klenow or large proteolytic cleavage fragment of DNAPEc1 contains the polymerase and 3' exonuclease activity but lacks the 5' nuclease activity. The Stoffel fragment of DNAPTaq (DNAPStf) lacks the 5' nuclease activity due to a genetic manipulation which deleted the N-terminal 289 amino acids of the polymerase molecule [Erlich *et al.*, *Science* 252:1643 (1991)]. WO 92/06200 describes a thermostable DNAP with an altered level of 5' to 3' exonuclease. U.S. Patent No. 5,108,892 describes a *Thermus aquaticus* DNAP without a 5' to 3' exonuclease. However, the art of molecular biology lacks a thermostable DNA polymerase with a lessened amount of synthetic activity.

5 The present invention provides 5' nucleases derived from thermostable Type A DNA polymerases that retain 5' nuclease activity but have reduced or absent synthetic activity. The ability to uncouple the synthetic activity of the enzyme from the 5' nuclease activity proves that the 5' nuclease activity does not require concurrent DNA synthesis as was previously reported (Gelfand, *PCR Technology, supra*).

10 The description of the invention is divided into: I. Detection of Specific Nucleic Acid Sequences Using 5' Nucleases; II. Generation of 5' Nucleases Derived From Thermostable DNA Polymerases; III. Detection of Specific Nucleic Acid Sequences Using 5' Nucleases in an Invader-Directed Cleavage Assay; IV. A Comparison Of Invasive Cleavage And Primer-Directed Cleavage; V. Fractionation Of Specific Nucleic Acids By Selective Charge Reversal; VI. Invader™-Directed Cleavage Using Miniprobcs And Mid-Range Probes; VII. Signal Enhancement By Tailing Of Reaction Products In The Invader™-Directed Cleavage Assay ; VIII. Improved Enzymes For Use In Invader™-Directed Cleavage Reactions

15 **I. Detection Of Specific Nucleic Acid Sequences Using 5' Nucleases**

20 The 5' nucleases of the invention form the basis of a novel detection assay for the identification of specific nucleic acid sequences. This detection system identifies the presence of specific nucleic acid sequences by requiring the annealing of two oligonucleotide probes to two portions of the target sequence. As used herein, the term "target sequence" or "target nucleic acid sequence" refers to a specific nucleic acid sequence within a polynucleotide sequence, such as genomic DNA or RNA, which is to be either detected or cleaved or both.

25 Figure 1A provides a schematic of one embodiment of the detection method of the present invention. The target sequence is recognized by two distinct oligonucleotides in the triggering or trigger reaction. It is preferred that one of these oligonucleotides is provided on a solid support. The other can be provided free. In Figure 1A the free oligo is indicated as a "primer" and the other oligo is shown attached to a bead designated as type 1. The target nucleic acid aligns the two